

Review

2'-Carbohydrate modifications in antisense oligonucleotide therapy: importance of conformation, configuration and conjugation

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Abstract

The 2'-position of the carbohydrate moiety has proven to be a fertile position for oligonucleotide modifications for antisense technology. The 2'-modifications exhibit high binding affinity to target RNA, enhanced chemical stability and nuclease resistance and increased lipophilicity. All high binding affinity 2'-modifications have C_{3'}-endo sugar pucker. In addition to gauche effects, charge effects are also important in determining the level of their nuclease resistance. Pharmacokinetic properties of oligonucleotides are altered by 2'-conjugates. For certain modifications (e.g., 2'-F), the configuration at the 2'-position, arabino vs. ribo, determines their ability to activate the enzyme RNase H. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: 2'-Carbohydrate modification; Antisense oligonucleotide therapy

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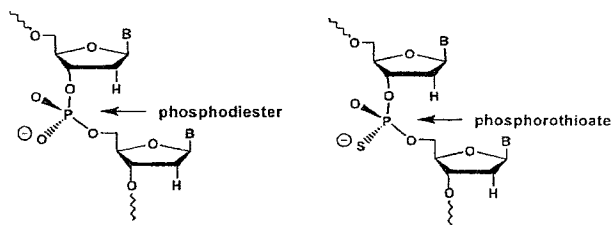
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1. Introduction

The first generation antisense oligonucleotides, 2-deoxyphosphorothioates (2'-H/PS, Fig. 1), have proven to be successful considering the number of compounds in the clinical trials for the treatment of a host of diseases, including cancer, inflammation and viral infection with this chemistry. The very first antisense compound entering the market ISIS-2922, "Vitravene", is a 2-deoxyphosphorothioate. The virtues of 2-deoxyphosphorothioates are numerous:

1. The ease of synthesis
2. Sufficient nuclease resistance for parenteral administration



phosphodiester: 2'-H/PO
prochiral linkage

2'-deoxyphosphorothioate: 2'-H/PS
chiral linkage, diastereomeric mixture

Fig. 1. Structural comparison of DNA-phosphodiester and DNA-phosphorothioate linkages.

3. Ability to activate RNase H to cleave RNA

4. Sufficient binding affinity to cellular proteins for uptake, absorption, and distribution [1]

On the other hand, like any other class of agents, they do have some limitations. The limitations of 2-deoxyphosphorothioates are:

1. The loss of binding affinity to the target (nearly -0.8°C for each nucleotide difference, presumably due to either the diastereomeric nature of the PS linkages or the higher molecular weight)
2. Inhibition of RNase H at high concentrations
3. Non-specific binding to proteins which leads to changes in clotting times and complement activation
4. No blood-brain barrier penetration
5. Poor oral bioavailability [2]

These limitations provide an opportunity for medicinal chemists to design and develop novel chemical modifications to improve the pharmacokinetic, pharmacodynamic and pharmacological properties of antisense compounds.

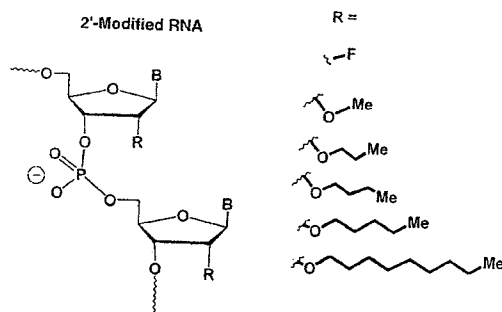
2. RNase (Ribonuclease H)

RNase H (Ribonuclease H) is an ubiquitous enzyme present in cells which hydrolyzes RNA in RNA–DNA duplexes [3]. In the context of antisense technology, the antisense strand has to play the role of DNA for RNase H to cleave the target mRNA. RNase H functions as an endonuclease, specifically cleave RNA in RNA–DNA duplexes, require divalent cations and generate products with 5'-phosphate and 3'-hydroxyl termini. The best characterized of the prokaryotic enzymes is *Escherichia coli* RNase H1 [4–7]. Recently Wu et al. reported the cloning and expression of a his-tag labeled RNase H from human cells and it is referred to as Human RNase H1 [8,9]. RNase H interacts with the RNA–DNA duplexes via the minor groove of the helix formed [4,10–13].

1. Second generation of chemical modifications: carbohydrate 2'-modifications

The second generation chemical modifications to improve the efficacy of an antisense oligonucleotide are designed to increase the binding affinity to target NA, to enhance the nuclease resistance, to improve cellular absorption and to modulate the protein binding of oligonucleotides by controlling the number of phosphorothioate linkages, i.e., ability to replace PS linkages with the native PO linkages without sacrificing either the nuclease resistance or the favorable pharmacokinetic properties of 2'-deoxyphosphorothioates [14–16].

Among the different sites available for chemical modification in a nucleoside building block, the 2'-



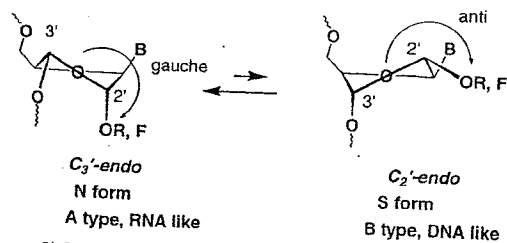
Binding Affinity: -F > -OMe > -O-Propyl > -O-Butyl > -O-Pentyl > -O-Nonyl

Nuclease Resistance: -O-Pentyl > -O-Propyl > -OMe > -F

Fig. 3. The effect of various 2'-modifications on target binding affinity and nuclease resistance.

position of the carbohydrate moiety [14] has proven to be the most valuable for various reasons:

1. 2'-Modification with an electronegative substituent can confer an RNA-like C3'-endo conformation to the antisense oligonucleotide. Such a preorganization favors an RNA like conformation [17–19] and greatly improves the binding affinity to the target RNA (Fig. 2);
2. 2'-Modification provides nuclease resistance to oligonucleotides even as a PO internucleotide linkage [20];
3. 2'-Modification provides chemical stability against potential depurination conditions either during synthesis or after administration as the drug;
4. 2'-Modification changes the lipophilicity of the oligonucleotides which relates to favorable pharmacokinetic properties;
5. These modifications will reside in the minor groove of the RNA-antisense oligonucleotide duplexes formed.



2'-OR, 2'-F favor C_{3'}-endo (O/O or O/F prefer gauche)

Fig. 2. Effect of 2'-electronegative substituents on the pentafuran conformational equilibrium.

Among the possible 2'-modifications, 2'-O-alkyls, 2'-O-alkyls with glycol ether linkages, 2'-F and 2'-O-aminoalkyls have been studied for their pharmacokinetic, pharmacodynamic and pharmacological properties. Within 2'-O-alkyls, with increase in size of the alkyl chain, the binding affinity [21] drops from 2.0°C to -1.0°C while the nuclease resistance increases with increase in alkyl chain [20] the O-pentyl group (as PO) exhibiting nuclease resistance similar to 2'-deoxyphosphorothioates (Fig. 3). The 2'-F modification, which locks the sugar conformation

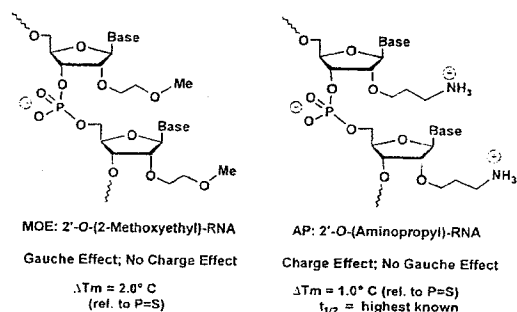


Fig. 4. Structures of 2'-O-MOE and 2'-O-AP modifications.

in a very high C3'-endo conformation (C3'-endo $> 90\%$) offers the greatest increase in binding affinity (+3.0/nucleotide linkage) [22]. Unfortunately, this high-affinity modification does not offer any resistance to nucleases as a phosphodiester (PO). It requires the phosphorothioate backbone (PS) to exhibit sufficient nuclease resistance.

4. 2'-O-MOE RNA and 2'-O-AP RNA

Among the 2'-modifications well studied at Isis Pharmaceuticals, two modifications stand out in terms of binding affinity to target RNA and nuclease resistance. These are 2'-O-(methoxyethyl) or 2'-O-MOE modification [23–25] and 2'-O-(aminopropyl) or 2'-O-AP modification [26] (Fig. 4).

The 2'-O-MOE modification offers +2°C increase in binding affinity/modification compared to the first generation of 2'-deoxyphosphorothioate drug compounds [27]. This modification as a phosphodiester (PO) linkage, exhibits nuclease resistance (measured as the half-life of the full-length oligonucleotide, $t_{1/2}$) at approximately the same level as a 2'-deoxyphosphorothioate (2'-H/PS) modification.

The 2'-O-aminopropyl (2'-O-AP-RNA) modification exhibits the highest nuclease resistance compared to PS (6 to 8 times better) and a modest increase in T_m (+1°C increase in binding affinity/modification) [26]. This improved nuclease resistance is due to the cationic alkyl chain (termed 'charge effect') present at the 2'-position. The amino group in the 2'-O-substituent has a pK_a in the range of 9–10 and can therefore be expected to be protonated at physiological pH. The cationic group decreases susceptibility of oligonucleotides to nucleases [26,28,29].

5. Mechanisms of action

5.1. RNase H-dependent mechanism of action of 2'-modified oligomers: gapmer technology

While the 2'-modifications offer increased binding and high nuclease resistance, they fail to activate RNase H for cleaving the target RNA after hybridization. This is presumably due to the formation of RNA–RNA-like hybrids rather than RNA–DNA hybrids needed for RNase H cleavage. This limitation has been overcome by the development of gapmer technology [30–32] in which chimeric oligonucleotides with the placement of 2'-modifications only at the terminal ends ('wings'), leaving a 2'-deoxyphosphorothioate gap – usually 6 to 10 bases long – ('Gap') in the middle for RNase H recognition and cleavage (Fig. 5).

5.2. RNase H-independent mechanism of action of 2'-modified oligomers: consequences of direct high-affinity binding to the target: inhibition of translation

What happens when a fully 2'-modified oligonucleotide having high-binding affinity to the target mRNA is used as the antisense construct? Obviously, this molecule cannot elicit RNase H activity. However, it can interfere with other metabolic processes associated with the mRNA (splicing or translation).

There are two reports of an RNase H-independent 2'-modified antisense oligonucleotides exhibiting

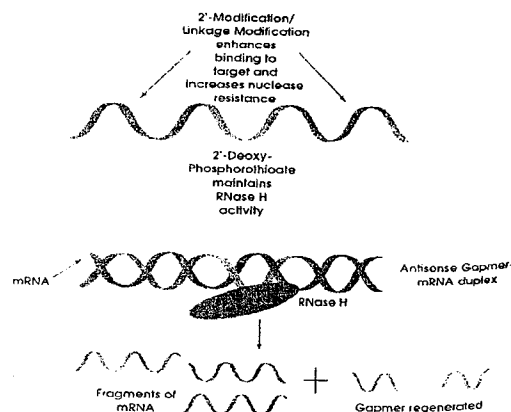


Fig. 5. Oligomer technology.

antisense activity due to translation arrest. The first one is seen in targeting 5'-cap region of human ICAM-1 transcript in HUVEC cells with a series of uniformly 2'-O modified 20-mer oligonucleotides which differ in binding affinity to the target RNA and nuclease resistance [33]. The 2'-O-MOE/PO oligomer demonstrated the greatest activity with an IC_{50} of 2.1 nM ($T_m = 87.1^\circ\text{C}$) and its PS analog had an IC_{50} of 6.5 nM ($T_m = 79.2^\circ\text{C}$). The unmodified parent oligonucleotide, (2'-H/PS compound), which can activate RNase H, exhibited an $IC_{50} = 41$ nM. Interestingly, the highest affinity oligomer (2'-F/PS) is not the most active one as it has an $IC_{50} = 8$ nM. The inhibition of protein expression by the RNase H-independent oligonucleotides was due to selective interference with the formation of the 80S translation initiation complex. The other metabolic processes such as splicing and transport of the transcript RNA formed were not affected by the 2'-modification. Another example is found in the case of human HCV as the target [34] where an uniformly modified 2'-O-MOE/PO phosphodiester antisense oligonucleotide ($T_m > 90^\circ\text{C}$) complementary to the initiator AUG codon reduced HCV core protein levels without reducing HCV RNA levels.

5.3. RNase H-independent mechanism of action of 2'-modified oligomers: consequences of direct high-affinity binding to the target: modulation of splicing

Yet another mechanism by which uniformly modified oligonucleotides can interfere with gene expression is via modulation of splicing events [35]. Modification of splicing in the dystrophin gene in cultured *mdx* muscle cells by uniformly modified 2'-O-Me modified phosphorothioates has been recently demonstrated. An antisense 2'-O-Me oligonucleotide complementary to the 3'-splice site of intron 22 induces dystrophin expression at the sarcolemma of transfected *mdx* myotubes. The mutant exon 23 of *mdx* dystrophin is skipped following transfection with an antisense 2'-O-Me modified phosphorothioate complementary to the 3' splice site of intron 22. Other research groups have reported similar observations in modulating splicing as a potential mechanism of controlling gene expression [36–38].

6. Important 2'-modifications

6.1. 2'-O-MOE RNA: unique antisense properties

Among the 2'-modifications, 2'-O-MOE-RNA forms duplexes with RNA that are 2°C more stable on average per modification than the corresponding 2'-H/PS-DNA-RNA hybrids [23–25, 27]. This modification also shows strong selfpairing properties [39]. The higher RNA affinity is accompanied by a significantly enhanced protection against nuclease degradation, comparable to that of 2'-H/PS-DNA [14,23–25]. Both features could help overcome the current pharmacodynamic, pharmacokinetic and safety-profile limitations encountered with phosphorothioate oligodeoxynucleotides (2'-H/PS-DNA). Thus, favorable RNA hybridization and less PS content may allow reduced dosing frequencies and permit the use of shorter antisense oligonucleotides. Higher stability of the antisense oligonucleotide/RNA complex lead to RNase H-independent downregulation of the targeted message through a translation arrest mechanism [33]. In addition, such antisense oligonucleotides might improve the chances for future oral administration of antisense therapeutics [40,41] and a reduction of the number of PS-linkages with retained or enhanced nuclease resistance should lead to lower immune stimulation and toxicity [42–47].

6.2. Structural origins of the improved antisense properties of 2'-O-MOE modification

Egli et al. [39,48] have rationalized the extraordinary nuclease stability and binding affinity of 2'-O-MOE-RNA by studying crystal structures of 2'-O-MOE-RNA molecules. The origins of the promising antisense properties displayed by 2'-O-MOE-RNA, have been determined by studying high-resolution crystal structures of

1. a palindromic DNA oligomer GCGTA-T_{MOE}ACGC having single '2'-O-MOE modification [48];
2. a completely 2'-O-MOE-modified RNA duplex, the first for an RNA molecule carrying a chemical modification on every residue [39].

In the first structure, the stabilizing 2'-O-MOE

substituent as well as the modified furanose ring (C3'-endo pucker) are conformationally preorganized for an A-form duplex. The conformation of the torsion angles around the ethyl C-C bonds in the side chain fall into the syn-clinal conformation. The resulting conformation is compatible with the minor groove topology in an A-form RNA duplex. The orientation of the side chain is further constrained by the coordination of water molecules involving O2', O3' and the ether oxygen. The water molecule lies within 3 Å of these three atoms. Such a hydrogen bonding would significantly contribute to the preorganization of the modified nucleoside into an A form. This complexation will also inhibit nucleases from cleaving the phosphate below the O3' atom (Fig. 6).

The recent structural results [39] with a fully modified 2'-O-MOE dodecamer CGCGAAUUCGCG are consistent with the higher RNA affinity of 2'-O-MOE-RNA compared with DNA. The sugars are locked in the C3'-endo conformation, as expected for any RNA mimic. As mentioned earlier, in the case of the 2'-O-alkyl modifications, longer substituents destabilize the duplex formed between the modified strand and the RNA target [21]. However, conformational preorganization with 2'-O-MOE-RNA includes both sugar moiety and the substituent and the gauche effect in 2'-O-modifications is extended. The observed conformational preferences of the 2'-O-moiety also prevail in the single-stranded state, providing an entropic advantage for pairing.

A further feature of 2'-O-MOE-RNA established by the crystal structures is the extensive hydration of the minor groove and backbones. Up to three first-shell water molecules can be stabilized by the 2-O moiety, assisted by bridging and non-bridging phosphate oxygens. The chelate-like trapping of water molecules between oxygen acceptors of substituent

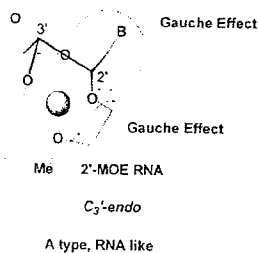


Fig. 6. Conformational preorganization of 2'-O-MOE RNA due to multiple gauche effects and hydration.

and backbone suggests an important role of water in the overall stability of 2'-O-MOE-RNA. In A-RNA, the 2'-hydroxyl groups can also stabilize water bridges between sugar and base and sugar and phosphate moieties in the minor groove [49]. By comparison, the hydration shells of phosphate groups, sugars and bases overlap only weakly in B-DNA [50].

2'-H/PS-DNAs showed relatively poor permeability in an *in situ* single-pass perfusion model that was used to assess the absorption of oligonucleotides in various segments of the rat intestine [41]. Conversely, 2'-O-MOE oligoribonucleotides with PO backbones had 2.5- to 10-fold increased permeability [41]. Paracellular absorption, believed to be the dominant route for uptake of oligonucleotides, is influenced by the size, charge and hydrophilicity of an antisense compound and correlates strongly with water flux and intercellular tight junction diameter. The highly improved uptake of 2'-O-MOE oligonucleotides relative to 2'-H/PS oligonucleotides is likely a consequence of the different backbone polarities and is consistent with the extensive hydration of the 2-O substituents.

The precise origins of nuclease resistance of 2'-O-MOE oligomers are not fully borne out even by this crystallographic study. Steric hindrance is a likely cause of the improved resistance observed with 2'-O-MOE-RNA. Thus, the 2'-O-MOE is similar to 2'-O-butyl in steric bulk. The intricate water network between the 2-O substituent and bridging and non-bridging phosphate oxygens may enhance steric hindrance. Stably bound water molecules could also interfere with phosphoryl transfer or could alter the reactivity of the phosphodiester moiety.

The lower toxicity of the 2-O-MOE modification, relative to 2'-H/PS-DNA may arise from the reduced avidity of 2'-O-MOE-RNA for serum protein binding as a result of its enhanced hydration.

Interestingly, molecular dynamics simulations of an 2'-O-MOE-RNA single strand furnished a rigid structure that closely resembles the conformation of the 2-O strand in the duplex state [51]. The structure and physical properties of 2'-sugar substituted O-(2-methoxyethyl) nucleic acids have been studied using molecular dynamics simulations [51]. Nanosecond simulations on the duplex 2'-O-MOE-[CCAACGTTGG]-r[CCAACGUUGG] in aqueous solution have been carried out using the particle mesh Ewald method.

od. Parameters for the simulation have been developed from ab initio calculations on methoxyethyl fragments in a manner consistent with the AMBER 4.1 force field database. The simulated duplex is compared with the crystal structure of the self-complementary duplex d[GCGTAT_{MOE}ACGC]2, which contains a single modification in each strand. Structural details from each sequence were analyzed to rationalize the stability imparted by substitution with 2'-O-(2-methoxyethyl) side chains. Both duplexes have an A-form structure, as indicated by several parameters, most notably a C3' endo sugar pucker in all residues. The simulated structure maintains a stable A-form geometry throughout the duration of the simulation. The presence of the 2' substitution appears to lock the sugars in the C3' endo conformation, causing the duplex to adopt a stable A-form geometry. The side chains themselves have a fairly rigid geometry with *trans*, *trans*, *gauche*+/- and *trans* rotations about the C2'-O2', O2'-Cα', Cα'-Cβ' and Cβ'-OCH₃ bonds, respectively.

5.3. What factors contribute to the extraordinary nuclease stability of 2'-O-AP RNA? The 'charge effect'

As mentioned above, the 2'-O-aminopropyl (2'-O-AP-RNA) modification [26], even as a diester (PO) exhibits the highest nuclease resistance compared to 5'-S oligonucleotides, due to the existence of positive charge on this group (the 'charge effect'). The increase in resistance is not a steric effect, since the propyl or longer pentyl modifications do not offer such an increase in nuclease resistance. Similarly the sugar conformation (C3'-endo population) does not seem to contribute to this improved stability because other C3'-endo substituents such as -F, -O-allyl do not exhibit such a robust stability. The side chain of 2'-O-AP-RNA has an amino group ($K_a=9.4$) and protonated at physiological pH. This positive charge can displace the metal ion cofactors at the nuclease active sites [52] of the nucleases, contributing to the high nuclease resistance. Other cationic oligonucleotide analogs have also been shown to contribute to increase in nuclease resistance [28,53,54]. However, the 2'-O-aminopropyl (2'-O-AP-RNA) modification lacks the *gauche* effect of the -O-

CH₂-CH₂-O- present in 2'-O-MOE-RNA linkage and this compromises the binding affinity of this modification. The lack of *gauche* effect minimizes the entropic advantage of the preorganization to form RNA-like duplex.

7. Going beyond 2'-MOE and 2'-AP modifications

To improve the pharmacokinetic and pharmacodynamic properties of 2'-modified oligomers even further by incorporating the attractive features of 2'-O-MOE (improved binding affinity due to the *gauche* effect) and 2'-O-AP modifications (nuclease resistance due to the charge effect), we are synthesizing many modifications, some of which are discussed below (2'-O-AOE and 2'-O-DMAOE, Fig. 7).

In designing the 2'-O-AOE and 2'-O-DMAOE modification we desired to maintain the following features:

1. An electronegative atom at 2'-connecting site, which is absolutely necessary for C_{3'}-endo conformation via O_{4'}-O_{2'} *gauche* effect (results in increase in binding affinity)
2. *Gauche* effect of the 2'-substituent -O-CH₂-CH₂-O- (results in increase in binding affinity/nuclease resistance)
3. Restricted motion around N-O bond as in the natural product calicheamicin, which will lead, to conformational constraints in side chain
4. Lipophilicity of the modification which relates to protein binding/absorption properties of oligonucleotides

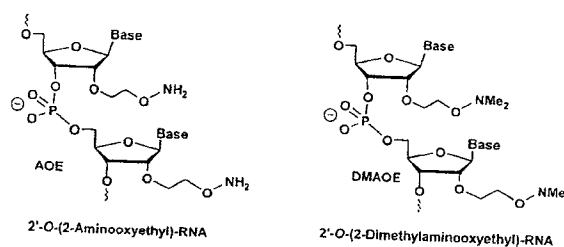


Fig. 7. Structure of 2'-O-AOE and 2'-O-DMAOE RNA modifications.

7.1. 2'-O-AOE (aminooxyethyl)

This modification [55] exhibits same binding affinity and same nuclease resistance as 2'-O-MOE RNA. This is because of the maintenance of gauche effect and the resulting favored preorganization. The oxy-amino group is not expected to be protonated at physiological conditions.

7.2. 2'-O-DMAOE (dimethylaminooxyethyl)

2'-O-DMAOE modification [56] shows higher binding affinity and higher nuclease resistance than 2'-O-MOE. The maintenance of gauche effect and favored preorganization causes the binding affinity advantage. Also, the improved steric effect (compared to 2'-O-MOE and 2'-O-AOE modifications) may be contributing to the enhanced nuclease resistance. This modification supports both an RNase H-dependent (as a gapmer) and an RNase H-independent mode of action in biological assays. Message walks have been started with this modification in several new targets to choose the appropriate target site which is optimal for this modification. Thus the 2'-O-DMAOE-RNA exhibits the attractive features of both 2'-O-MOE-RNA and 2'-O-AP-RNA although it also lacks the charge effect of the 2'-O-AP modification.

8. Oligonucleotide conjugates as carbohydrate modifications: aminolinkers and thiol linkers at the 2'-position for conjugation chemistry

The 2'-O-alkylation methodology developed at Isis

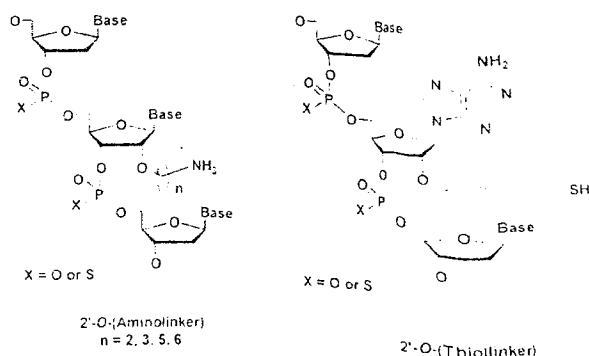


Fig. 8. Structures of 2'-O-aminolinkers and a 2'-O-thiol linker

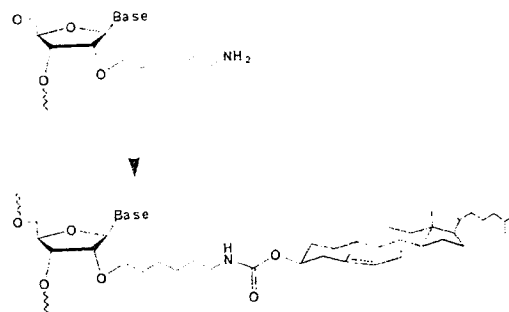


Fig. 9. Cholesterol conjugated oligonucleotide via 2'-O-aminolinker.

had led into various aminolinkers and thiol linkers at the 2'-position (Fig. 8). The length of the tether and the nucleophilic end are versatile in this strategy.

A 2'-O-hexylthiotriaryl adenosine phosphoramidite was synthesized and incorporated into oligodeoxynucleotides. These oligonucleotides possess the lipophilic 2'-O-hexylthiotriaryl group at preselected positions. Upon treatment with silver nitrate solution, a free thiol group was generated which was further functionalized. This thiol tether offered a convenient nucleophile for conjugation of various pendant moieties that reside in the minor groove of the duplex formed [57,58].

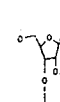
Similarly, several 2'-aminolinkers have been developed and numerous conjugates have been synthesized at this position via these linkers [53,56,28,59–63].

Finally, in addition to the above chemical modifications, the carbohydrate 2'-position has been used as a site for conjugating other ligands via an amino group to improve the antisense properties of oligonucleotides. For example, the 2'-cholesterol conjugate, changes the biodistribution and pharmacokinetic properties of antisense oligonucleotides dramatically [64]. The modification is very lipophilic and it substantially increased the fraction of the drug accumulated by the liver (Fig. 9).

9. Oligonucleotide crosslinking via 2'-modifications

In addition, an efficient site-specific crosslinking reaction between two carbohydrate residues present in two complementary DNA sequences has been developed using 2'-aminolinkers (Fig. 10). One oligo

STRAND:



3' GCCT

5' CGCAT

Fig. 10.
abasic s

deoxy
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CAGC
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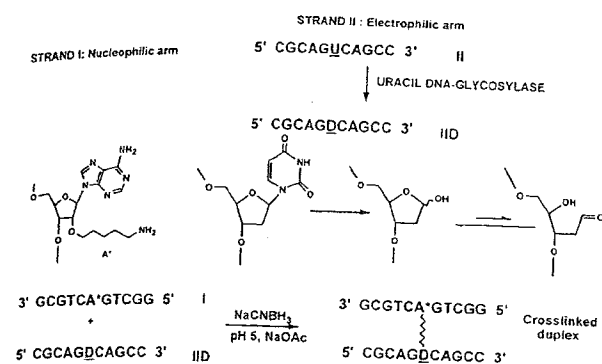


Fig. 10. Site-specific crosslinking of oligonucleotides using the abasic site and 2'-O-(aminopentyl) linker.

deoxynucleotide, 5'd(GGCTGA*CTGCG)3', carries an amino nucleophile tethered to the 2'-hydroxyl of an adenosine residue (A*) via a five-carbon tether. The target electrophile is an abasic site generated in the complementary sequence, 5'd(CGCAGD-CAGCC)3' (D represents the deoxyribose). The abasic site was generated from the oligonucleotide carrying deoxyuridine residue 5'd(CGCAGUCAGCC)3' by treatment with uracil-DNA glycosylase enzyme. The crosslinking reaction was carried out by a reductive amination reaction in >95% yield [65]. The resultant crosslink is well-accommodated within the duplex without any distortion, enhancing the duplex stability considerably (Fig. 11).

10. 2'-Modified oligonucleotides with ability to activate RNase H

It was pointed out earlier that 2'-modified oligonucleotides are not capable of activating RNase H in oligonucleotide: RNA hybrid. However, recently Damha et al. [66] have reported that hybrids of RNA and arabinonucleic acids (ANA) and 2'-deoxy-2'-fluoro-D-arabinonucleic acid (2'F-ANA) are substrates of RNase H (Fig. 12).

In their study on hybridization properties of arabinonucleic acid (ANA), the 2'-stereoisomer of RNA based on D-arabinose instead of the natural D-ribose (2'-arabino-OH)/RNA hybrids had a lower value of T_m , compared to the control DNA/RNA hybrids. This destabilization is presumed to derive from steric interference by the -C2'-OH group, which is oriented

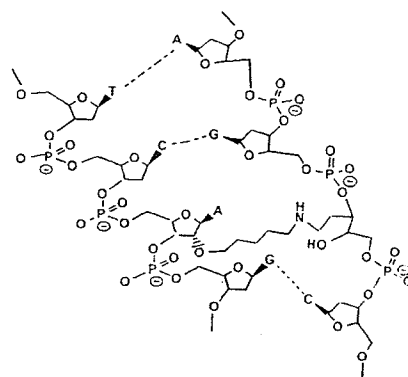


Fig. 11. Chemical structure of the crosslink formed between a 2'-O-(aminopentyl) tether and the abasic site.

into the major groove of the helix, causing slight local deformation (unstacking). Replacing the ara-2'-OH group by a 2'-F atom resulted in a marked increase in duplex melting temperature. The 2'F-ANA/RNA duplex had a higher value of T_m compared to the corresponding hybrids formed by ANA, PS-DNA, and DNA. The significantly higher value of T_m for 2'F-ANA/RNA relative to that for ANA/RNA hybrids may reflect reduced steric interactions of fluorine atoms (versus OH groups), whereas the higher value of T_m of 2'F-ANA/RNA relative to that DNA/RNA hybrids may result from a higher pre-organization state of 2'F-ANA relative to DNA (a -2'-F atom is expected to stabilize the C2'-endo sugar pucker).

The similar helical conformations of ANA (2'F, or 2'OH)/RNA and DNA/RNA hybrids have led the authors to compare their susceptibility to cleavage by HIV-1 RT-associated RNase H and *E. coli* RNase H. They observed both HIV-RT and *E. coli* RNase H were able to degrade RNA hybridized to arabinonucleic acids. However, ANA (2'-OH-ANA)/RNA

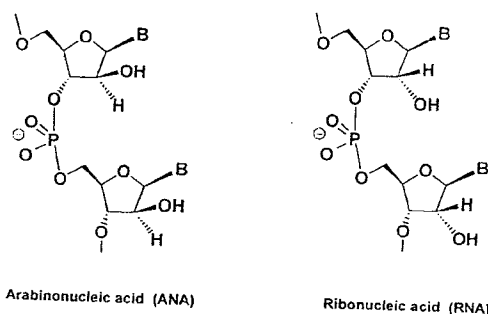


Fig. 12. Structures of ANA and RNA.

hybrids were less susceptible to cleavage than (2'-F-ANA)/RNA, which can be attributed to the lower thermal stability of the former class of duplexed (i.e., less duplex present). The authors conclude that the ability of RNase H to degrade RNA in ANA/RNA hybrids (2'-F or 2'-OH) may result from (a) the similarity of structure of these hybrids to that of the normal DNA/RNA substrate and (b) the fact that the 2'-OH or F substituents of the arabinose sugar ring projects into the major groove of the helix, at a site where it should not interfere with the binding and catalytic processes of RNase H. In addition, they show that arabinonucleic acids exhibit more nuclease-resistance to serum and cellular nucleases compared to DNA strands although less than phosphorothioate (2'-H/PS) oligonucleotides.

Venkateswarlu and Ferguson [67] studied the effects of C2'-substitution on arabinonucleic acid structure and conformation by a computational study and compared their results to those of Damha et al. An analysis of the sugar puckers of the ANA (2'-F) and ANA (2'-OH) strands indicates that both adopt a C2'-endo geometry, consistent with the *ab initio* model calculations. While the standard arabinose sugars favor southern pucker phase angles of ~ 145 – 162 (C2'-endo), the values are slightly more northern and varied toward the O4'-endo domain (~ 73 – 144) in the fluoro-substituted strand. This difference is most likely due to the formation of an internal hydrogen bond between the C2'-hydroxyl group and the C5'-oxygen of the arabinose sugar that essentially 'locks' the conformation in the C2'-endo form. A comparison of the minor groove widths (as estimated by interstrand phosphate distances) of the ANA(2'-F):RNA, ANA(2'-OH):RNA, and DNA:RNA structures shows all three share intermediate values (14–15 Å) when compared to ideal A- or B-form geometries. This may explain the RNase H activity of ANA:RNA hybrids reported by Damha. It has also been suggested that the steric composition of the minor groove may be an important factor for RNase H recognition. For most ribose-based antisense nucleotides, C2'-substitutions project into the minor groove, potentially blocking recognition by RNase H. The ANA C2'-substituents, however, adopt the opposite stereochemistry and occupy steric space in the major groove of the duplex. Although this suggests that most ANA C2'-modifi-

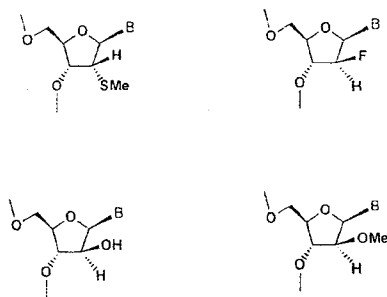
cations would project away from the minor groove, they must also maintain C2'-endo sugar pucker for RNase H activation.

The sugar puckers of the ANA(2'-OH) strand were found to adopt a more southern C2'-endo conformation when compared with the fluoro or methoxy derivatives. This may explain the observed decrease in stability and RNase H activity previously reported for ANA(2'-OH):RNA complexes when compared with analogous ANA(2'-F):RNA hybrids by Damha et al. The theoretical study further indicate that the ANA(2'-OH) strand is more rigid due to the formation of an intramolecular hydrogen bond between the C2'-hydroxyl and the C5'-oxygen of the arabinose sugar. This most likely preorganizes the ANA strand to a more B-like conformation, leading to less favorable ANA–RNA interactions in the A-like hybrid geometry.

11. RNase H activation with 2'-ribo modification? 2'-SMe modification

In a different report, Ferguson et al. [68] studied the structural properties of DNA:RNA duplexes containing 2'-O-methyl and 2'-S-methyl ribo-substitutions in the ribose sugar by molecular dynamics. Molecular dynamics simulations of 2'-SMeDNA:RNA, 2'-OMe-RNA:RNA and standard DNA:RNA hybrids in explicit water indicate that the nature of the C2'-ribo substituent has a significant influence on the macromolecular conformation. The preference for C3'-endo puckering follows the following trend: 2'-OMe-RNA > DNA:RNA > 2'-SMe-DNA. These results are further corroborated using *ab initio* methods. Both gas phase and implicit solvation calculations show the C2'-OMe group stabilizes the C3'-endo conformation while the less electronegative SMe group (where there is absence of gauche effect) stabilizes the C2'-endo conformation when compared to the standard nucleoside. The macromolecular conformation of these nucleic acids also follows an analogous trend with the degree of A-form character decreasing as OMe-RNA:RNA > DNA:RNA > SMe-DNA:RNA.

Whereas the O-Me substitution leads to a slight expansion of the minor groove width when compared to the standard DNA:RNA complex, the S-



2'-Modifications claimed to activate RNase H

Fig. 13. 2'-Modifications in the ribo- and arabino-configurations, which are claimed to have RNase H activity.

methyl substitution leads to a general contraction (~ 0.9 Å). In addition to the minor groove width changes, the calculations also point to potential differences in the steric makeup of the minor groove. The *O*-methyl group points into the minor groove while the *S*-methyl is directed away towards the major groove. Essentially, the *S*-methyl group has flipped through the bases into the major groove as a consequence of C2'-endo puckering. Based on these results the authors have predicted that the 2'-SME substitution will elicit RNase H activity (Fig. 13). The *O*-methyl group may also play some role in deactivating the enzyme. The *S*-methyl substitution destabilizes oligonucleotide:RNA complexes by $\sim 1.5^\circ$ per base pair when compared with native DNA:RNA hybrids [69,70]. Unfortunately, no experimental RNase H data is yet available for 2'-SMe-containing derivatives.

12. Conclusions and perspectives

The 2'-position of the carbohydrate residue continues to be a valuable site for chemical modifications for antisense technology and other genome-based drug discovery efforts. Many chemical processes, which are not directly presented here, have evolved for simpler and efficient methods of these key modifications [71]. In vitro and in vivo pharmacology evaluations and correlation with pharmacokinetic changes are emerging from these novel chemical modifications. Analytical chemistry of modified oligonucleotides before and after biological administration of antisense oligonucleotides with techniques

such as capillary gel electrophoresis and mass spectrometry help to determine the purity as well as the in vivo fate of these complex molecules. Large-scale synthesis is becoming a tangible reality for both first generation and second generation e.g., 2'-*O*-MOE-RNA antisense oligonucleotides. Nucleic acid chemists and biologists alike are beginning to understand the structure-biological activity in terms of basic physical-organic parameters such as the gauche effect, the charge effect and conformational constraints. Synthesis of chimeric *designer* oligonucleotides bringing the attractive features of different modifications to a given antisense oligonucleotide sequence to generate synergistic interactions is forthcoming [72]. These advances along with the potential availability of complete human genome sequence information promise a bright future for the widespread use of nucleic acid based therapeutics.

It is pertinent to conclude this review by pointing out that there are three 2'-*O*-MOE modified oligomers in Isis Pharmaceuticals' product pipeline: ISIS 13312 against HCMV, a oligomer against TNF- α as anti-inflammation drug and another oligomer against VLA-4 also as an anti-inflammation drug.

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Abstract

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